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Biochemistry

Covalent binding and hemolytic activity of complement proteins

(inactivation by amines)

S. K. LAW, N. A. LICHTENBERG, AND R. P. LEVINE

The James S. McDonnell Department of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110

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ABSTRACT **We report the inactivation of the third component of complement (C3) by hydroxylamine. C3 hemolytic and covalent binding activities decline with identical kinetics, demonstrating a direct correlation between the two activities. We conclude that covalent, surface-bound C3b is hemolytically active. The inactivation of C3 is first order with respect to hydroxylamine. We also studied C3 inactivation with [^{14}C]methylamine. The inactivation corresponds quantitatively with the labeling of C3 in the C3d domain. The data obtained support the following hypothesis: there is an internal thioester within C3 which becomes highly reactive on activation to C3b, and C3b binds to receptive surfaces by transfer of the acyl function of the thioester to a hydroxyl group on the receptive surface. This proposed model for the reaction of C3 with receptive surfaces also applies to C4, which binds to membrane surfaces covalently and is able to be inactivated by hydroxylamine and methylamine. C5, on the other hand, is not inactivated by treatment with the amines.**

C3 (185,000 daltons), the third complement protein, is composed of two polypeptides, C3α (110,000 daltons) and C3β (75,000 daltons), crosslinked by disulfide bonds (1, 2). C3 exists in serum as an inert molecule until foreign cells activate the complement system. The central step in complement activation involves cleavage of C3 to C3b and the binding of C3b, via its labile binding site, to the foreign cells (for review of C3 and the complement system, see refs. 3 and 4). We previously showed that the interaction between the labile binding site of C3 and receptive surfaces (RS) is covalent (5) and that the labile binding site lies in the C3d fragment of the C3bα polypeptide (6). Subsequent experiments (7) established that the bond between C3b and RS is an ester of the form C3b-CO-O-RS. It remained to be proven, however, that the covalently bound C3b on RS is responsible for the C3b-mediated functions of cytolysis and opsonization.

In this study, we treated C3 with primary amines and compared the rate of decay of C3 hemolytic activity with the rate of loss of surface-binding activity. We find both rates to be identical, indicating that both activities are causally related. We also present data to support our hypothesis (7) that there is an internal ester bond within C3 and that C3b binds to RS by the transfer of the acyl group of the internal ester to a hydroxyl group on the RS. C4 and C5 were also treated with hydroxylamine and methylamine. C4, which binds covalently to membrane surfaces (8), behaves like C3 in these experiments. C5, which does not bind covalently to cell surfaces (8), is not inactivated by hydroxylamine and methylamine.

MATERIALS AND METHODS

Sera, Complement Proteins, and Cells. Functionally pure guinea pig C1 and C2 were isolated from guinea pig serum (Pel-Freez) (9). Human C3, C4, and C5 were isolated from fresh human plasma (10, 11). Human C6, C7, C8, and C9 were purchased from Cordis Laboratories (Miami, FL). Sheep erythrocytes (E) were sensitized with rabbit anti-sheep hemolysin (Microbiological Associates, Bethesda, MD) to form EA (9). EA bearing C1 and C4 (EAC14) were purchased from Cordis Laboratories. A reagent lacking C3 activity (R3) was prepared from human plasma (12). C4-deficient guinea pig serum was a gift from J. P. Atkinson. C3b was prepared by treating C3 with trypsin, 1000:1 (wt/wt), for 5 min at 37°C (13). C3d was prepared by the digestion of C3 with trypsin, 200:1 (wt/wt), at pH 7.5 and 37°C for 4 hr followed by separation from C3c by standard gel filtration (Sephadex G-100) and ion-exchange (DEAE-Sephacel) techniques.

Chemicals and Reagents. [^{14}C]Methylamine·HCl, ^{125}I, ^{125}I-labeled Bolton–Hunter reagent, Aquasol, Protosol, and Omnifluor were purchased from New England Nuclear. Sepharose-bound lactoperoxidase and trypsin were obtained from Worthington. Zymosan was purchased from ICN.

Radioiodination of Proteins. C3 was labeled with ^{125}I either by the Sepharose-bound lactoperoxidase method (14) or with the ^{125}I-labeled Bolton–Hunter reagent (15). The specific radioactivities of C3 labeled by the two methods (C3*) were 100,000 cpm/μg and 50,000 cpm/μg, respectively. Less than 1% of the hemolytic activity was lost by the radioiodination procedure.

Treatment of C3, C4, and C5 with Hydroxylamine. C3, C4, or C5, at 1–5 mg/ml, was dialyzed into 0.1 M sodium phosphate (pH 7.5). Hydroxylamine·HCl (1 M) in 0.1 M sodium phosphate (pH 7.5) was added to give the desired hydroxylamine concentration. The mixture was incubated at a selected temperature between 19°C and 37°C. At specific times, aliquots of the reaction mixture were removed and dialyzed against 5 mM Veronal buffer/0.15 M NaCl, pH 7.5, at 4°C to remove excess hydroxylamine. In experiments in which covalent binding activity was also determined, radioactively labeled C3 (C3*) was used. When the kinetics of inactivation of the proteins was measured as a function of hydroxylamine concentration, all samples were adjusted to the same protein concentration and conductivity by the addition of buffer (0.1 M sodium phosphate) or buffer with NaCl.

Treatment of C3, C4, and C5 with Methylamine. The treatment of C3, C4, and C5 with methylamine was the same as the treatment with hydroxylamine except that the reaction was carried out in Veronal buffer/0.15 M NaCl, pH 8.0.

In experiments in which radioactive methylamine was used, [^{14}C]methylamine·HCl in ethanol, at 0.1 mCi/ml (1 Ci = 3.7 $\times$ 10^{10} becquerels), was dried in a test tube under nitrogen at

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Abbreviations: C, complement; EA, sheep erythrocytes sensitized with rabbit anti-sheep hemolysin; EAC14, EA bearing active C1 and C4; R3, a reagent lacking C3 activity prepared from plasma; DGVB^{2+} buffer, 2.5 mM Veronal buffer, pH 7.5/75 mM NaCl/2.5% dextrose/0.05% gelatin/0.15 mM $CaCl_2$/0.5 mM $MgCl_2$; ZX, zymoscan particles with surface-bound guinea pig alternative pathway C3 convertase; RS, receptive surfaces for C3b.

37°C. An appropriate amount of protein solution, at 1–5 mg/ml, in Veronal buffer/0.15 M NaCl, pH 8.0, was added to the [^{14}C]methylamine to give a final amine concentration of 5 mM. Unreacted [^{14}C]methylamine was removed by dialysis.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. NaDodSO$_4$/polyacrylamide gel electrophoresis was carried out in the Laemmli system (16) under reducing conditions, with slab gels having a gradient of 6–12% polyacrylamide. In experiments in which radiolabeled polypeptides were analyzed, the appropriate bands were cut out and radioactivity was measured.

Assay for Hemolytic Activity of C3, C4, and C5. C3 samples were serially diluted (1:2 or 1:3) in 0.2 ml of DGVB^{2+} buffer (2.5 mM Veronal buffer, pH 7.5/75 mM NaCl/2.5% dextrose/0.05% gelatin/0.15 mM $CaCl_2$/0.5 mM $MgCl_2$). EAC14 (0.1 ml; 1×10^8 cells per ml) was added followed by 0.2 ml of 1:20 diluted R3 reagent. The mixture was incubated for 60 min at 37°C; 0.5 ml of DGVB^{2+} buffer was added, and the cells were sedimented by centrifugation. The degree of hemolysis was determined by measuring the absorbance of the supernatant at 414 nm. One unit of C3 hemolytic activity was defined as the inverse of the dilution at which 63% lysis was obtained. C3-specific hemolytic activity is the C3 hemolytic activity divided by the C3 protein concentration.

The hemolytic assay for C4 is similar to that of C3 except that EA were used instead of EAC14 and C4-deficient guinea pig serum (diluted 1:100) was used in place of R3.

For measurements of C5 hemolytic activity, C5 samples were serially diluted in 0.2 ml of DGVB^{2+} buffer. EAC14 (0.1 ml; 1×10^8 cells per ml) was added followed by 0.1 ml of a solution containing C2, C3, C6, and C7, each at 100 units/ml. The mixture was incubated at 30°C for 30 min; 0.1 ml of a solution containing C8 and C9, each at 100 units/ml, was added, and the mixture was further incubated at 37°C for 60 min. C5-specific hemolytic activity was determined as for C3 and C4.

For the comparison of C3 hemolytic and covalent binding activities, an accurate determination of specific hemolytic activity was essential. C3*, at 4000 cpm/μg, was used such that it carried significantly measurable radioactivity at a concentration that gave 30–75% hemolysis in the assay. The C3 concentration in each tube was determined by its radioactivity, and the C3 hemolytic activity was calculated by the formula $\mu = -\log(1 - y)$, in which y is the fraction of cells lysed. In this way, the protein concentration and hemolytic activity of C3 were measured in the same tube. An accurate determination of the specific hemolytic activity is thus obtained without the errors involved in serial dilutions.

Covalent Binding Assay for C3*. Zymosan particles with surface-bound guinea pig alternative pathway C3 convertase (ZX) were prepared as described (17). Samples of C3*, at about 250 μg/ml and 2000 cpm/μg, were incubated with 4 mg of ZX in 0.5 ml of DGVB^{2+} buffer. The experiment was done in quadruplicate for each C3* sample. As a control, duplicate samples of C3* were incubated with zymosan. All incubations were carried out at 37°C for 30 min, after which 0.1 ml of 20% (wt/vol) NaDodSO$_4$ was added to stop the reaction. The mixture was further incubated at 90°C for 3 min or at 37°C for 30–90 min. The radioactivity of each sample was determined in order to measure the total amount of C3* in each reaction mixture. The zymosan particles were washed twice with 1% NaDodSO$_4$ at room temperature. The radioactivity associated with the particles was measured. The C3* covalent binding activity is expressed as the percentage of bound radioactivity after NaDodSO$_4$ washes relative to total input C3* radioactivity. In a typical experiment, the C3* sample not treated with hydroxylamine gave ≈3% C3b* binding.

Conversion of C3 to C3b. This assay is performed in conjunction with the covalent binding assay. The supernatant from the reaction of ZX (and zymosan) with C3* was analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The three bands, C3α, C3bα, and C3β, were cut out and their radioactivity was determined. The amount of conversion is defined by the ratio of radioactivity, C3bα/(C3α + C3bα).

Other Assays. Protein concentrations were determined by the method of Lowry *et al.* (18). Gel slices containing ^{14}C-labeled polypeptides were solubilized by incubation in a solution of 3% (vol/vol) Protosol and 0.4% Omnifluor in toluene at 37°C for 24 hr before scintillation spectrometry.

RESULTS

Correlation Between Covalent Binding of C3b and Its Hemolytic Activity. C3* (1–5 mg/ml) was treated with hydroxylamine (0.15 M and 0.25 M, pH 7.5, 21°C) for various lengths of time. After removal of hydroxylamine by dialysis, the C3 hemolytic activity and covalent binding activity were measured. The results (Fig. 1*A*) show that the two activities declined in parallel with pseudo-first-order kinetics. The first-order rate constants for the inactivation were 0.0075 min^{-1} at 0.15 M hydroxylamine and 0.013 min^{-1} at 0.25 M hydroxylamine. Similar results were obtained when the measurements were performed with 0.05–0.3 M hydroxylamine and temperatures of 19–37°C. Identical results were obtained for C3 ^{125}I-labeled at its tyrosine residues or labeled with the ^{125}I-labeled Bolton–Hunter reagent at its amino groups.

Inactivation of C3 by Hydroxylamine. How does hydroxylamine inactivate C3? NaDodSO$_4$/polyacrylamide gel electrophoresis of hydroxylamine-inactivated C3 show that the size of the C3 polypeptides was not altered (Fig. 1*B*, lanes a and b). Hydroxylamine does not inhibit C3 cleavage by the $\overline{C3B}$ enzyme. The supernatants from the incubation of C3* with ZX and zymosan were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The amount of conversion of C3 to C3b, ≈35%, was the same for C3 samples inactivated to different extents by hydroxylamine (Fig. 1 *A* and *B*, lanes c–g).

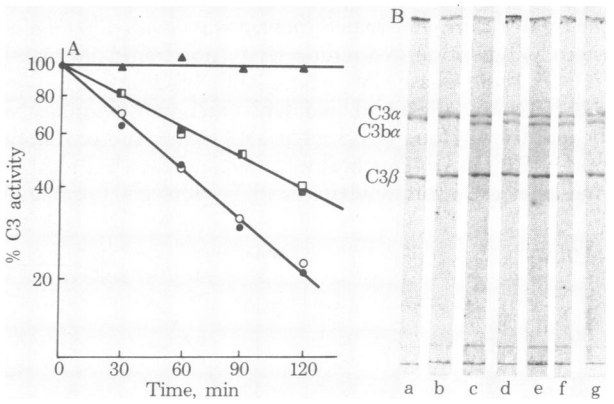

FIG. 1. Kinetics of inactivation of C3 with hydroxylamine. The reaction was carried out at pH 7.5 and 21°C. (*A*) Inactivation of C3 hemolytic activity (■ and ●) and covalent binding activity (□ and ○) at 0.15 M (■ and □) and 0.25 M (● and ○) hydroxylamine. The conversion of C3 to C3b as a function of time of treatment with 0.25 M hydroxylamine (▲) is also shown. All activities are expressed as a percentage of the C3 sample treated for zero time with hydroxylamine. (*B*) NaDodSO$_4$/polyacrylamide gel electrophoresis of C3 and C3b samples: lane a, C3; lane b, C3 treated with 0.25 M hydroxylamine for 2 hr; lanes c–g, supernatants of the incubation of ZX and C3 samples treated with 0.25 M hydroxylamine for 0 (lane c), 30 (lane d), 60 (lane e), 90 (lane f), and 120 (lane g) min.

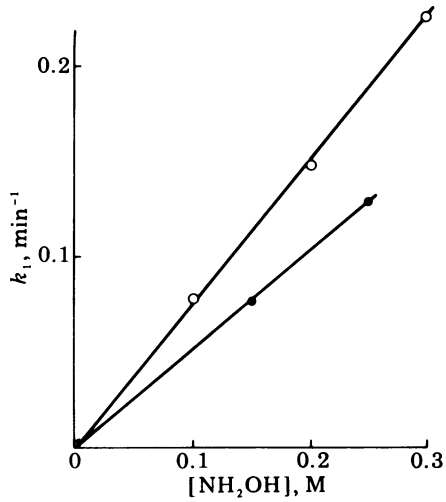

FIG. 2. Plot of k_1 for the inactivation of C3 by hydroxylamine at 21°C (●) and 25°C (○) against [NH_2OH].

The rates of inactivation of C3 at different hydroxylamine concentrations were plotted against the hydroxylamine concentration (Fig. 2). The data give a straight line passing through the origin, indicating that the inactivation is first order with respect to hydroxylamine. The k_2 value for the reaction is 0.051 M^{-1} min^{-1} at 21°C and pH 7.5. Data from another experiment in which C3 was inactivated at three different hydroxylamine concentrations at pH 7.5 and 25°C are also included in Fig. 2. The k_2 value for the reaction at 25°C is 0.075 M^{-1} min^{-1}.

Inactivation of C3 by Methylamine. Similar experiments were performed with methylamine instead of hydroxylamine. At 5 mM methylamine at pH 8.0 and 37°C, 50% of the C3 was inactivated in 41 min; k_2 of the reaction is 3.4 M^{-1} min^{-1} (data not shown). C3, when incubated at the same pH and temperature in the absence of methylamine, was fully active after 24 hr.

Inactivation and Labeling of C3 by [^{14}C]Methylamine. C3 was treated with 5 mM [^{14}C]methylamine for 140 min at 37°C; this reduced the specific hemolytic activity to 7% of an untreated control. $NaDodSO_4$/polyacrylamide gel electrophoresis of the treated sample revealed that C3 was covalently labeled at the α polypeptide. No radioactivity was found associated with C3β. Fluid-phase C3b, whose labile binding site has decayed (19), was also incubated with [^{14}C]methylamine. No radioactivity was found associated with either its α or β polypeptide (Fig. 3A). Furthermore, when the [^{14}C]methylamine-labeled C3 was incubated with trypsin (1%) to generate

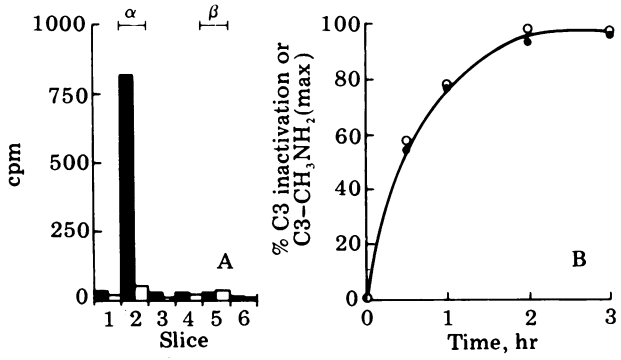

FIG. 3. Inactivation and labeling of C3 by [^{14}C]methylamine. (*A*) $NaDodSO_4$/polyacrylamide gel electrophoresis of $^{14}CH_3NH_2$-treated C3 (■) and C3b (□). (*B*) Quantitative correlation between the inactivation (●) and labeling (○) of C3 with $^{14}CH_3NH_2$.

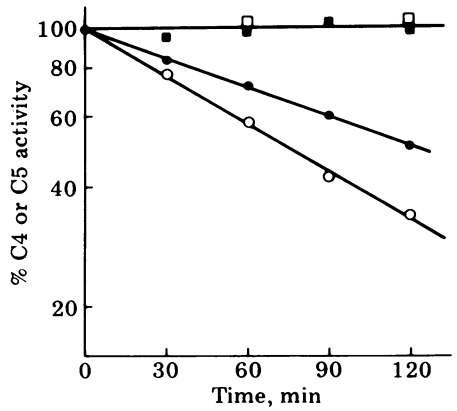

FIG. 4. Treatment of C4 (○ and ●) and C5 (■ and □) with 0.5 M hydroxylamine at pH 7.5 and 21°C (○ and □) and 5 mM methylamine at pH 8 and 21°C (● and □). The hemolytic activity of the proteins is shown.

C3c and C3d and the peptides were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, all the radioactivity was found on C3d (data not shown).

The rate of the labeling of C3 by [^{14}C]methylamine at pH 8.0 and 37°C was also studied. The incorporation of [^{14}C]-methylamine into C3 follows the equation: C3–$CH_3NH_2(t)$ = C3–CH_3NH_2(max)$(1 - e^{-k't})$, in which C3–CH_3NH_2(max)† is the maximal amount of [^{14}C]methylamine incorporated into C3 on prolonged incubation (>4 hr) and k' is the first-order rate constant of the reaction. The fraction in percent of C3–$CH_3NH_2(t)$ with respect to C3–CH_3NH_2(max) was plotted against time (Fig. 3*B*); the degree of labeling correlated directly with the degree of inactivation of the C3 hemolytic activity.

Inactivation of C4 with Hydroxylamine and Methylamine. The sensitivity of C4 to hydroxylamine and methylamine is well known (21). Fig. 4 shows the time courses of these reactions. The reaction of C4 with hydroxylamine and methylamine was qualitatively identical to that of C3, with only quantitative differences in the absolute rates: the k_2 values for the inactivation of C3 and C4 are 0.051 M^{-1} min^{-1} and 0.018 M^{-1} min^{-1}, respectively (hydroxylamine, pH 7.5 and 21°C) and 3.4 M^{-1} min^{-1} and 7.2 M^{-1} min^{-1}, respectively (methylamine, pH 8.0 and 37°C). In experiments in which [^{14}C]methylamine was used to inactivate C4, the label was found in the C4α polypeptide only (data not shown).

Treatment of C5 with Hydroxylamine and Methylamine. Treatment of C5 with 0.5 M hydroxylamine (pH 7.5 and 20°C for 2 hr) did not significantly decrease C5-specific hemolytic activity. When C5 was treated with 5 mM [^{14}C]methylamine (pH 8.0 and 37°C for 2 hr), no loss of hemolytic activity was detected (Fig. 4) and there was no detectable labeling of C5 by [^{14}C]methylamine.

† The need to define a term C3–CH_3NH_2(max) is due to the fact that not all C3 molecules in different C3 preparations are active. It is not uncommon for C3 to lose its activity during the isolation procedure or in storage (or both) (20). This term, C3–CH_3NH_2(max), can therefore be used as an index of quality for different C3 preparations. In the ideal case, C3–CH_3NH_2(max) should correspond to one CH_3NH_2 molecule per C3 molecule. In experiments reported in this paper, three different C3 preparations were used with C3–CH_3NH_2(max) values corresponding to the CH_3NH_2:C3 ratios of 0.34, 0.78, and 0.98. The quality of C3 used in these experiments, however, does not affect the interpretation of the data obtained, for most of the experiments presented have been repeated with the C3 preparation whose C3–CH_3NH_2(max) value corresponds to a CH_3NH_2:C3 ratio of 0.98.

DISCUSSION

In this paper, we present evidence showing that the covalent binding of C3 to RS (5, 7) correlates directly with its hemolytic activity. In addition, we have obtained further evidence to support our hypothesis (7) that C3 binds by a transesterification mechanism.

The hydroxylamine inactivation of both the hemolytic and covalent binding activities of C3 demonstrates the relationship between the two activities. If the covalently bound C3b molecules are responsible for the hemolytic activity, the two activities should be inactivated with identical kinetics; the data in Fig. 1 demonstrate that this is the case. Furthermore, hydroxylamine does not cleave C3 or interfere with its conversion to C3b (Fig. 1). Hydroxylamine, therefore, must block the covalent binding process that occurs within fractions of a second after C3 is cleaved into C3b (22). This action causes a concomitant loss in hemolytic activity. Thus, we conclude that the covalently bound C3b molecules are active in the hemolytic process.

We previously proposed (7) the presence of an internal ester within C3, probably protected from the aqueous environment in a hydrophobic pocket. When C3 is activated to C3b, the ester becomes exposed and either reacts with a nearby surface-bound hydroxyl group by the transfer of the acyl function or is hydrolyzed. We found that the inactivation of C3 is first order with hydroxylamine, indicating that only one hydroxylamine molecule is required to inactivate C3. These data support our hypothesis (7) in which we predict one NH_2OH-sensitive site on C3—the site occupied by the internal ester.

Observations by Janatova *et al.* (23) also support the concept of the internal ester and clarify the chemical nature of the internal bond. They found that C3 lacks free sulfhydryl groups in its native state, but that a free sulfhydryl group appears after inactivation by hydroxylamine. The same sulfhydryl group is also found on C3b. These results suggest that the proposed internal ester is a thioester (23); this idea is particularly attractive considering that the transfer of an acyl group from a thioester to an oxygen ester is energetically favorable.

The inactivation of C3 by methylamine also leads to the appearance of a free sulfhydryl on the C3 molecule (data not shown; also see ref. 24). The use of [^{14}C]methylamine to inactivate C3 results in the labeling of C3, and the degree of labeling directly correlates with the loss of hemolytic activity (Fig. 3*B*). Furthermore, the label is found in the C3α polypeptide (Fig. 3*A*).

The k_2 value of 3.4 M^{-1} min^{-1} for the methylamine inactivation of C3 is calculated with respect to the total methylamine concentration, which is $[CH_3NH_2]$ plus $[CH_3NH_3^+]$. With 10.64 as the pK_a value for methylamine, the free base concentration $[CH_3NH_2]$ equals 11.4 μM at pH 8.0 and a total methylamine concentration of 5 mM. Therefore, the k_2 value for the inactivation reaction of C3 with the free base is 1360 M^{-1} min^{-1}, an extremely high value, three orders of magnitude faster than values obtained from the *n*-butaminolysis of ethyl thioacetate (25) and ethyl *p*-nitrothiobenzoate (26). In order to account for the extreme sensitivity of C3 to methylamine, we suggest that there exist on C3, in the vicinity of the thioester, one or more catalytic groups that cause the elevated rate of reaction. The kinetics of the inactivation of C3 by hydroxylamine differs from that of the hydroxylaminolysis of thioesters in aqueous solutions: whereas the inactivation of C3 is first order with hydroxylamine, that of the hydroxylaminolysis of thioesters contains a second-order dependence term, with the second molecule of hydroxylamine playing a catalytic role (27). The observed first-order kinetics of the hydroxylamine inactivation of C3, therefore, also leads one to predict the presence of hydroxylamine-like catalytic group(s) in the vicinity of the thioester.

Thus far, all the results obtained from experiments involving the use of either hydroxylamine or methylamine to inactivate C3, as presented in this paper and by Janatova *et al.* (23) and Tack *et al.* (24), support the model shown in Fig. 5. (*1*) There is an internal thioester within C3, which becomes exposed or activated when C3 is cleaved to C3b. (*2*) The thioester reacts with a hydroxyl group on RS by a transesterification mechanism, resulting in the binding of C3b to the RS via an ester bond. (*3*) Abortive C3b molecules are those whose exposed internal thioesters hydrolyze before they can react with surface-bound hydroxyl groups. (*4*) The hydroxylaminolysis and methylaminolysis of the thioester, which affects neither the C3 primary structure nor the ability to C3 to interact with the $\overline{C3B}$ enzyme, prevents the molecule from covalently binding to receptive surfaces. (*5*) The loss of covalent binding capacity leads to a loss in hemolytic activity of C3b.

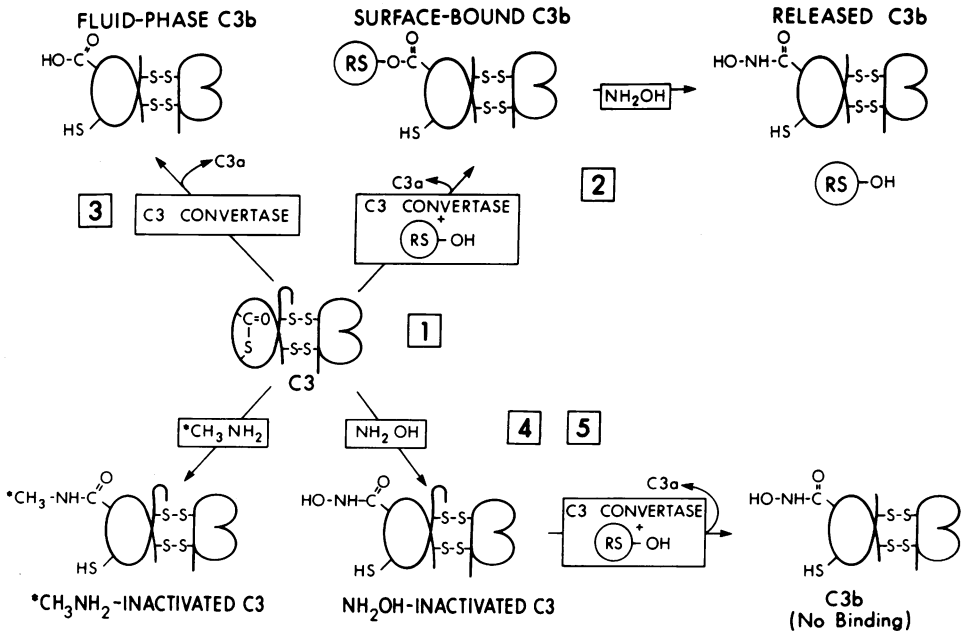

FIG. 5. Active site of C3 (see text).

We have also studied the homologous proteins C4 and C5 (3, 28). C4, which binds covalently to cell-surface molecules (8), is also inactivated by hydroxylamine and methylamine. The difference in the rates of reaction of C3 and C4 with hydroxylamine and methylamine suggests that there may be a difference in the accessibility of the hypothetical thioesters in C3 and C4. Whereas C3 is more sensitive to hydroxylamine, C4 is more susceptible to methylamine inactivation. C5, which neither interacts covalently with cell surfaces (8) nor possesses a detectable thioester, is not affected by hydroxylamine or methylamine under the conditions that inactivate C3 and C4.

The hypothetical internal thioester within C3 and C4 may not be unique to those proteins. α_2-Macroglobulin, which regulates the activities of certain proteases (29), can also be inactivated and labeled with [^{14}C]methylamine (30), and a radioactive peptide has been isolated in which the radioactivity is carried in a γ-glutamylmethylamide residue (30). Furthermore, the interaction between α_2-macroglobulin and proteases is probably covalent in nature because the α_2-macroglobulin–protease complexes are stable in $NaDodSO_4$ (31). The inactivation of C3 with [^{14}C]methylamine has also been studied by Tack *et al.* (24), who have isolated a peptide having a radioactive γ-glutamylmethylamide residue. The amino acid sequences of the peptides from α_2-macroglobulin and C3 show substantial homology (24), and it is likely that C4 may also possess a similar peptide. The presence of an energetic internal thioester within C3, C4, and α_2-macroglobulin might be the means by which each of them interacts covalently with a range of substrates: C3 (5) and C4 (8) bind indiscriminately to different kinds of cell surfaces, and α_2-macroglobulin binds to a variety of proteases (29). It is our belief that these three proteins are representative of a larger class of proteins, each having an internal thioester and each capable of mediating various generalized functions following covalent binding to their respective substrates.

Note Added in Proof. Since this paper was submitted, a paper by Pangburn and Müller-Eberhard has appeared (32) reporting results similar to those described here.

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